

## Material and Methods

**Bacterial strains, growth conditions and antibiotic susceptibility testing.** *S. aureus* N315 was cultured using tryptic soy broth (TSB) or tryptic soy agar (TSA) at 37 °C. Antibiotics ampicillin and chloramphenicol were used for plasmid selection at a concentration of 100 µg/ml and 20 µg/ml, respectively. Arylomycin M131 was synthesized as previously described (1, 2) and used at a concentration of 4 µg/ml corresponding to 4× the MIC of wild type *S. aureus* N315. Susceptibility to arylomycin M131 was performed as previously described (3). For strains containing plasmids, susceptibility testing was performed in the presence of chloramphenicol for plasmid selection. Evolved arylomycin-resistant isolates were obtained as previously described (3).

**Whole genome sequencing of an evolved arylomycin M131 resistant isolate of *S. aureus* N315.** Approximately 570 ng total RNA was fragmented using a Covaris S2 ultrasonicator (5% duty cycle, Intensity = 3, 200 cycles per burst, 40 s). Fragmented DNA was cleaned up using 0.5× Ampure beads and eluted in 0.1× TE. DNA was then prepared into a sequencing library using the New England Biolabs NEBNext® Ultra™ DNA Library Prep Kit for Illumina following the manufacturer's instructions with 6 cycles of PCR. The library was then sequenced on an Illumina MiSeq using 2 × 300 paired-end reads.

**Strain construction.** The *isaA* deletion strain was created as previously described (4). Briefly, a ~1 kb region upstream of the *isaA* gene was PCR amplified using primers IsaA\_Up\_For (5'-GGAGGTACCGCAGTATTGATAATTGGTACA) and IsaAUp\_Rev (5'-ACATTACTTTTATTATTATGAAGGAATTACATAGTAAAAATCCTCCAGT). A ~1 kb region downstream of the *isaA* gene was PCR amplified using primers IsaA\_DWN\_For (5'-ACAATTATTACTGGAGGATTTTTTACTATGTAATTCCTTCATAATAAATAAAAG) and IsaA\_DWN\_Rev (5'-TCCGGAATTCGTAACAGAATCATTAAAGATATGC). The upstream and downstream PCR products were amplified using IsaA\_UP\_For and IsaA\_DWN\_Rev to create a ~2 kb product using overlap extension. The 2 kb product was introduced into pIMAY by restriction digest (KpnI and EcoRI) and ligation. The resulting deletion plasmid was transformed into SA30B (Lucigen, Wisconsin USA). 4 µg of DNA isolated from SA30B was used to transform the deletion plasmid into *S. aureus* N315 by electroporation as previously described (3) and plated on TSB plus chloramphenicol at 30 °C. Single recombination of the deletion plasmid was achieved by subculturing 1:100 in TSB plus chloramphenicol at 43 °C for 24 h twice, followed by plating on prewarmed TSA plus chloramphenicol at 43 °C. For the second recombination of the plasmid, a single colony was inoculated in TSB without selection, grown for 24 h and subcultured 1:100 repeatedly until plasmid loss occurred. Plasmid loss was assessed by plating on TSA without selection and patching colonies on TSA with and without chloramphenicol. Chloramphenicol-susceptible colonies were subjected to colony PCR with primers flanking the *IsaA* gene, IsaAver\_For (5'-GTGTTGATTGCTTTTTTAATTGCG) and IsaAver\_Rev (5'-CCAATTTCTATGGGAAGAGCT). Amplification of a ~1200 bp fragment indicated that the *isaA* gene was present while a smaller ~500 bp fragment indicated that *isaA* was deleted. To create *AyrA*(R233K) $\Delta$ *ayrBC* and *IsaA*(K2Q) $\Delta$ *ayrRABC*, deletion plasmids previously created for  $\Delta$ *ayrBC* and  $\Delta$ *ayrRABC* (5) were introduced into *AyrA*(R233K) and *IsaA*(K2Q), and the above protocol was followed with successful deletions assessed by colony PCR using primers previously published (3).

**Plasmid Construction.** The plasmid pSK5630 was used to create all complementation vectors (6, 7). The template was genomic DNA isolated from *S. aureus* N315, unless otherwise stated. In all cases successful constructs were confirmed by DNA sequencing and plasmids introduced into N315  $\Delta$ *isaA* using electroporation as previously described (3).

$P_{isaA}$ -*isaA* and  $P_{isaA}$ -*isaA*(K2Q) were created by PCR amplification of the *isaA* gene using genomic DNA from wildtype and the *IsaA*(K2Q) mutant strain, respectively. 400 bp upstream of the translational start site of *isaA* (to include the *isaA* promoter) was included using the primers *IsaA*\_For (5'-ggaGGATCCGTGTTGATTGCTTTTAATTGCG) and *IsaA*\_Rev (5'-TCCTCTAGACTAGTGATGGTGATGGTGAGAGCCTCCACCGAATCCCCAAGCACCTAAAC). A His<sub>6</sub> tag with a Gly<sub>3</sub>Ser linker was added to the C-terminus of *isaA* alleles to allow for Western blot analysis. The amplified products were introduced into pSK5630 through restriction digest (BamHI and SmaI) and ligation.

All chimeric constructs were created by overlap extension.  $P_{isaA}$ -*BlaZ* was created using primers *IsaA*\_For and *BlaZ*promWT\_Rev (5'-AATTACAATTAATAAATATTAACCTTTTCAAAGTAAAAATCCTCCAGTAATAATTG) amplified the 400 bp promoter region of *isaA* and primers *BlaZ*FL\_For (5'-CTTACAATTATTACTGGAGGATTTTACTTTGAAAAAGTTAATATTTTAATTG) and *BlaZ*FL\_Rev (5'-GGTTTAGGTGCTTGGGGATTCTAGctagaGGGAA) amplified the ~850bp *blaZ* gene. To install the resistance conferring K2Q mutations primers *IsaA*\_For and *BlaZ*promK2Q\_Rev (5'-AATTACAATTAATAAATATTAACCTTTTCAAAGTAAAAATCCTCCAGTAATAATTG) were used to amplify the promoter region and *BlaZ*K2Q\_For (5'-CTTACAATTATTACTGGAGGATTTTACTTTGAAACAGTTAATATTTTAATTG) and *BlaZ*FL\_Rev to amplify the *blaZ* gene. In both cases primers *IsaA*\_For and *BlaZ*FL\_Rev were used to join the two fragments. The resulting ~1250 bp PCR products were cloned into pSK5630 using restriction digest (BamHI and SmaI) and ligation.

$P_{isaA}$ -*IsaA*<sub>sp</sub>*BlaZ*<sub>ec</sub> and  $P_{isaA}$ -*IsaA*(K2Q)<sub>sp</sub>*BlaZ*<sub>ec</sub> were created using the *IsaA*\_For primer with *IsaA*sp\_Rev (5'-ATATTTTTTTCTAAATCATTTAACTCTTTAGCGTGTGCTTGATGTCCT) using WT and *isaA*(K2Q)genomic DNA, respectively. The extracellular domain of *BlaZ* was amplified using *IsaA*sp\_FOR (5'-GCAGCAGGTACAGGACATCAAGCACACGCTAAAGAGTTAAATGATTTAGAAAA) and *BlaZ*his\_Rev (5'-TCCAAGCTTCTAGTGATGGTGATGATGGTGAGAGCCTCCACCAAATTCCTTCATTACACTCTT). To join the promoter/signal peptide and extracellular domain PCR products primers *IsaA*\_For and *BlaZ*his\_Rev were used for both WT and the K2Q mutant. The resulting PCR fragments were introduced into pSK5630 by restriction digest (BamHI and SmaI) and ligation. To install the non-cleavable proline mutation, the final constructs were subject to site directed mutagenesis using the primers *Pro*\_For (5'-CACACGCTCCTGAAGTAAACGT) and *Pro*\_Rev (5'-CTTGATGTCCTGTACCTGCTG).

All plasmids in which only the *IsaA* signal peptide was swapped were created using  $P_{isaA}$ -*isaA* as the template and the following primers:

$P_{isaA}$ -SA1754<sub>sp</sub>*IsaA*<sub>ec</sub> SA1754WT\_For (5'-GCTAAAACGATTGCTAAAGTTCCCGCAAGTATAGACTTTTTCATAGTAAAAAATCC) SA1754sp\_Rev (5'-ATCACCAGTAACTAATCTAGATAAAAAATGAGGCACAAGCTGCTGAAGTAAAC),  $P_{isaA}$ -SA1754(K2Q)<sub>sp</sub>*IsaA*<sub>ec</sub> SA1754K2Q\_For (5'-GCTAAAACGATTGCTAAAGTTCCCGCAAGTATAGACTTTTGCATAGTAAAAAATCC) and SA1754sp\_Rev,  $P_{isaA}$ -SceD<sub>sp</sub>*IsaA*<sub>ec</sub> SceDWT\_For (5'-AACCTACTGCTAATGATGATGCGAGTAATGTCTTTTTCATAGTAAAAAATCC) SceDsp\_Rev (5'-TAGGAATCGTAGCAGGAAATGCAGGTCACGAAGCCCATGCACTGAAGTAAAC),  $P_{isaA}$ -SceD(K2Q)<sub>sp</sub>*IsaA*<sub>ec</sub> SceDK2Q\_For (5'-AACCTACTGCTAATGATGATGCGAGTAATGTCTTTTTCATAGTAAAAAATCC) and SceDsp\_Rev, and  $P_{isaA}$ -AtpF(K2Q)<sub>sp</sub>*IsaA*<sub>ec</sub> and AtpFK2Q\_For (5'-ACTCAACGCCTCCAGCTGCACCAAGAACGAATACTTTTGCATAGTAAAAAATCCTC) AtpFK2Q\_Rev (5'-). The resulting ~7 kb linear full-length plasmids were DpnI treated overnight at 37 °C. PCR product (100 ng) was treated with T4 kinase for 30 min at 37 °C and ligated.

**RNA isolation and RT-PCR.** RNA isolation and conversion to cDNA was carried out as previously described (3). SYBR Select (Invitrogen) and a Bio-Rad CFX Connect real-time system were used for reverse transcription (RT)-PCR analysis using cDNA template (50 ng) and

the primers (625 nM) previously described in Table S2 of Craney, 2015 (3). The *gmk* gene was used to normalize gene expression and the parental strain *S. aureus* N315 was used to normalize gene expression between strains. Gene expression changes were measured in triplicate using the  $\Delta\Delta C_T$  method and CFX Manager 3.0 software (Bio-Rad).

**Western blot analysis.** The secretion of IsaA and BlaZ His-tagged alleles were assessed by western blot analysis. Cells were grown to an OD<sub>600</sub> of 0.6 in TSB plus chloramphenicol, pelleted by centrifugation and washed twice in sterile saline. After washing, the pellets were resuspended to an OD<sub>600</sub> of 0.6 in TSB plus chloramphenicol and split in two 20 ml cultures with arylomycin M131 added to one and the other serving as the DMSO only control. Cultures were grown at 37 °C for 30 min and cultures were again pelleted by centrifugation. The supernatant was removed to a fresh 50 mL conical tube and subjected to TCA precipitation as previously described (8, 9). Samples were run on 15% SDS PAGE gels and transferred to PVDF for subsequent western blotting. For detection, an HRP-conjugated primary antibody directed at the 6× His epitope was used at 1:5000 (Proteintech, IL, USA) with membranes incubated for 1 h, washed 3× for 15 min in PBST and visualized using Pierce™ ECL Western Blotting Substrate (ThermoFischer Scientific, MA, USA), followed by exposure to Amersham Hyperfilm ECL film (GE Healthcare Life Sciences, PA, USA).

#### References

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